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PHARMACEUTICAL TECHNOLOGY

PHARMACOKINETICS OF DICLOFENAC SODIUM AND PAPAVERINE HYDROCHLORIDE AFTER ORAL ADMINISTRATION OF TABLETS TO RABBITS

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Abstract: Non-compartmental pharmacokinetic analysis of diclofenac sodium (DIC) and papaverine hydrochloride (PAP) after oral administration of composed tablets to rabbits was developed. HPLC method for determination of DIC and PAP in rabbit plasma was developed and validated. Chromatographic separation of DIC, PAP and the IS was achieved on a Zorbax SB C18 5-µm column (150 mm × 4.6 mm) using methanol-water (55 : 45, v/v) as mobile phase at a flow rate of 0.8 mL/min. Pharmacokinetic analysis showed that oral administration of a tablet composed of DIC and PAP do not change the pharmacokinetic parameters such as MRT, MAT, Cl and bioavailability of the active substances compared with single administration of DIC and PAP after single dose.

Keywords: pharmacokinetics, diclofenac sodium, papaverine hydrochloride, rabbits, HPLC

Diclofenac sodium is a nonsteroidal anti-inflammatory drug (NSAID) that it used for the treatment of rheumatic diseases, minor and medium pain, and post surgery analgesia in medicine. Patients who are given formulations of diclofenac or other NSAIDs as a therapeutic strategy often suffer from the gastrointestinal tract complications (1-3). In order to decrease the adverse effects or to increase the therapeutic analgesic effect, diclofenac was orally administered in composed tablets comprising also misoprostol (4) and topically in a formulation comprising e.g., a spasmolytic agent - papaverine hydrochloride (5). In the literature can be found reports of a separate administration of diclofenac and papaverine for the relief of pain in patients with renal colic (6, 7). There are no reports of a formulation comprising diclofenac sodium and papaverine hydrochloride in a composed dosage form, therefore, we decided to prepare tablets consisting of DIC and PAP for oral administration for analgesic effect. Next, the tablets comprising the two drugs were patented (8).

In the literature, there are no reports regarding the relationship of pharmacokinetic parameters between DIC and PAP. Diclofenac is almost completely absorbed after oral administration, it is subjected to first-pass metabolism so that about 50% of the drug reaches the systemic circulation in the unchanged form. More than 99% is bound to plasma proteins, primarily to albumin (3, 9, 10). Diclofenac exhibits a terminal half-life of 1-2 h (3, 9). Metabolism of diclofenac is mediated by both glucuronidation and oxidative biotransformation (11). The oxidative metabolism of diclofenac is catalyzed by two enzymes of the cytochrome P450 family, namely CYP2C9 and CYP3A4 (12). Diclofenac is metabolized to 4'-hydroxydiclofenac and 5-hydroxydiclofenac, 3'-hydroxydiclofenac and 4',5-dihydroxydiclofenac (13, 14). They are then excreted in the form of glucuronide and sulfate conjugates, mainly in the urine (about 60%) but also in the bile (about 35%) and less than 1% is excreted as unchanged diclofenac (3).

Papaverine is completely absorbed by the gastrointestinal tract and metabolized principally by the liver. Papaverine in about 90% bound to plasma proteins. The biological half-life is reported to be between 1 and 2 h (3). The major route of biotransformation is O-demethylation to the corresponding

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phenolic metabolites (15). Several phenolic metabolites have been identified: 4'-desmethylpapaverine (which predominantes in most cell systems), 6-desmethylpapaverine, 7'-desmethylpapaverine, 4',6-didesmethylpapaverine and 3'-desmethylpapaverine (16-18). Papaverine is excreted in the urine, almost entirely as glucuronide-conjugated phenolic metabolites (3).

To test the pharmacokinetics and bioavailability of DIC and PAP given in a single formulation, it was necessary to develop a method for determination of DIC and PAP in rabbit plasma. According to the literature, several HPLC methods using different clean-up procedures, including direct injection, protein precipitation (19-21), solid-phase extraction (SPE) (22), liquid-liquid extraction (LLE) (23-26) and column switching (27) with UV (24-29), fluorimetric (20) or electrochemical detection (30) HPTLC (31) and HPLC-MS/MS (32), GC-MS (33, 34) were applied to determine diclofenac in animal or human plasma. Papaverine hydrochloride in biological fluids was assayed using HPLC (35-37), GLC (38), and GC (39) methods.

There are no data about pharmacokinetics of diclofenac sodium and papaverine hydrochloride after administration in composed formulation and there is no HPLC method for simultaneous determination of diclofenac sodium and papaverine hydrochloride levels in plasma. Therefore, the aim of this study was to examine the pharmacokinetics of DIC and PAP after oral administration of composed tablets to rabbits and develop and validate the HPLC method.

EXPERIMENTAL

Reagents and chemicals

Diclofenac sodium (DIC) was produced by Caesar and Loretz, GmbH, Hilden, Germany, papaverine hydrochloride (PAP) was purchased from Galfarm PPH, Cefarm Lublin, Poland, and phenacetin (IS) from POCh Gliwice, Poland. Polyvinylpyrrolidone K 22 (PVP), mannitol (M) and potato starch (PS) were the products of Merck, Germany. Chloroform was obtained from POCh Gliwice, Poland. Methanol and water (HPLC grade) were purchased from Merck, Germany and other reagents were of analytical grade.

Preparation of tablets

Composition and preparation of tablets (T) containing DIC and PAP were described in the patent (8). Tablets containing only one substance, DIC (T-DIC) or PAP only (T-PAP), were obtained in the same manner as described in this patent, but composition contained only one active substance.

One dose of tablets (T) consists of 50 mg DIC, 20 mg PAP and excipients such as 70 mg PVP, 70 mg M and 90 mg PS to obtain 300 mg of weight. One dose of tablets (T-DIC) consists of 50 mg DIC and excipients to obtain 280 mg of weight. One dose of tablets (T-PAP) consists of 20 mg PAP and excipients to obtain 250 mg of weight.

Tablets were obtained by direct compression of granules, which were previously prepared by wet granulation method. Granules were obtained by mixing and wetting of the powders with PVP solution until a mass of suitable consistency was obtained. Then, the wet mass was granulated using a

Table 1. Physical properties of prepared tablets.

Test	Results		
	T	T-DIC	T-PAP
Weight (mg) mean SD	300.54 ± 2.45	281.32 ± 1.55	249.68 ± 1.87
Thickness (mm) SD	4.02 ± 0.02	3.85 ± 0.01	3.8 ± 0.02
Disintegration time (min) SD	7 ± 2.5	6 ± 1.5	5 ± 2.1
Hardness (kG/mm^2), SD	0.105 ± 0.01	0.1 ± 0.01	0.105 ± 0.03
Friability (%)	0.09	0.22	0.16
Drug content (%) DIC, SD (%) PAP, SD	99.08 ± 1.17 100.05 ± 1.76	100.04 ± 3.93	97.35 ± 1.23

rotary granulator (Erweka, Germany) and a 1.6 mm mesh screen. Granules were dried in a hot air oven (Memmert INB-500) at 40°C for 1 h. The dried granules (moisture 3-5%) were sieved through a 1.6 mm mesh screen. The tablets were obtained from these granules in a press tableting machine (Erweka, Germany) with 9 mm concave punches.

Physical properties of tablets

The tablets were tested as per standard procedure for weight variation (n = 20), thickness (n = 20), hardness (n = 6), friability (n = 20), disintegration time (n = 6) and drug content (n = 10) (Table 1).

Weight uniformity test

For each formulation, twenty tablets were selected randomly and weighed together and their mean weight was calculated. Next, they were individually weighed using a weighing balance (Ohaus AV 513C, USA).

Tablet dimensions

Tablet diameter and thickness were measured using a Vernier Caliper (Digital Caliper 0-150 mm, Comparator).

Hardness test

Hardness of tablet was determined by using an Erweka tablet hardness tester (Erweka, Germany).

Friability test

An Erweka friabilator (Erweka, Germany) was used for the test. Twenty tablets were weighed, subjected to attrition at 25 rpm for 4 min and reweighed afterwards. The percentage loss in weight equivalent to friability was calculated from the equation below: Friability (%) = (loss in weight/initial weight) × 100.

Disintegration time

Disintegration time was measured by using an Erweka apparatus (Germany). Each of six tablets was put into a basket-rack in a vessel and was covered with a disk. After the apparatus had been turned on, the disintegration time of the tablets was observed.

Drug content analysis

Ten tablets from each series were selected at random, weighed together and the mean weight was determined. The tablets were crushed together and weighed exactly 300 mg (T), 280 mg (T-DIC) or 250 mg (T-PAP), when in powder form (n = 6), dissolved in methanol in a 50 mL volumetric flask, filtered using the Whatman filter and appropriately diluted with methanol. Drug content of DIC and PAP were analyzed by HPLC method published in earlier report (40).

Chromatographic conditions

The chromatographic separation of DIC, PAP and the IS (phenacetin) was achieved with a Perkin Elmer 200 HPLC system consisting of the 200 series pump, the 200 series autosampler equipped with a 200 µL loop, the 200 series UV/VIS detector, the 200 series vaccum degasser and a Zorbax SB C18 5-µm column (150 × 4.6 mm, Agilent, USA).

The mobile phase consisted of methanol and water (55 : 45, v/v), and the flow rate was 0.8 mL/min. The injection volume was 100 µL. The UV detection wavelengths at maximum absorbance of DIC and PAP at 280 nm and 238 nm, respectively, were chosen. The samples were injected twice because the Perkin Elmer 200 HPLC system enabled a detection for only one wavelength.

Calibration standards

Stock solutions of DIC (250 µg/mL) and PAP (100 µg/mL) were prepared in mobile phase solution (methanol-water, 55 : 45, v/v) and stored at 4°C wrapped in aluminum foil. The working solutions at concentrations of 0.5, 1.25, 2.5, 5, 12.5, 25 and 50 µg/mL of DIC and 0.5, 1, 2, 5, 10, 20, 40 and 60 µg/mL of PAP were prepared by serial dilution of DIC or PAP stock solutions in mobile phase. The IS working solution (20 µg/mL) was also prepared by dilution of the IS stock solution (100 µg/mL) with mobile phase.

The calibration standards were obtained by appropriate dilution of working solutions with mobile phase. The calibration standards were prepared on the same day at concentrations of 0.05, 0.125, 0.25, 0.5, 1.25, 2.5 and 5 µg/mL of DIC and 0.05, 0.1, 0.2, 0.5, 1, 2, 4 and 6 µg/mL of PAP, by spiking 1 mL blank rabbit plasma with 100 µL of DIC or PAP working solutions and 100 µL of IS working solution.

Sample and calibration standard preparation

Briefly, a 1 mL of blank rabbit plasma, spiked plasma (calibration standard sample) or pharmacokinetic study plasma sample was initially spiked with 100 µL of IS working solution and then 1.5 mL of 1 M hydrochloric acid was added. The mixture was shaken for the period of 10 min. To the obtained mixture, 3 mL of chloroform was added, shaken for 10 min and centrifuged at 3600 rpm for 10 min. The supernatant was separated and evaporated to dryness under nitrogen gas. The residue was reconstituted

with 400 µL of mobile phase and 100 µL aliquot of resulting solution was injected into HPLC apparatus. The samples setting into the vials were immediately assayed. Having obtained a chromatogram at a wavelength of 280 nm, 100 µL of the aliquot was injected once again and assayed at a wavelength of 238 nm for the assay of DIC and PAP, respectively.

Selectivity

The selectivity of the method was tested by comparing the chromatograms of blank plasma controls from six rabbits with that of plasma spiked with DIC, PAP and IS.

Sensitivity

The calibration standard at the lowest concentration, yielding a precision with relative standard deviations (RSD) less than 20% and accuracy within 20% of the nominal concentration, was considered to be the lower limit of quantification.

Calibration curve

Calibration standards at concentrations of 0.05, 0.125, 0.25, 0.5, 1.25, 2.5 and 5 µg/mL of DIC and 0.05, 0.1, 0.2, 0.5, 1, 2, 4 and 6 µg/mL of PAP (n = 2) were freshly prepared as described above and assayed on the same day. This assay was repeated for five consecutive days with freshly prepared calibration standards. The subsequent five calibration curves (y = ax + b), represented by the plots of the peak area of DIC to IS or PAP to IS (y) *versus* the concentration of the calibration standards (x), were generated and obtained by linear least-squares regression as the mathematical model.

Accuracy and precision

In order to validate the intra-day accuracy and precision, the samples of DIC (0.05, 0,25, 1.25 and 5 µg/mL) and PAP (0.05, 0.2, 2, 6 µg/mL) were freshly prepared and analyzed on the same day (n = 5). To validate the inter-day accuracy and precision, the intra-day accuracy and precision assay was repeated for three consecutive days. Data are presented in Table 2.

Accuracy was calculated by comparing the average concentration found to the known concentration, and was expressed in percentage as relative error. Precision was evaluated by calculating the RSD of measured concentrations at each level.

Recovery

The extraction recoveries of DIC and PAP from rabbit plasma at levels (0.05, 0.25, 1.25 and 5 µg/mL of DIC and 0.05, 0.2, 1, 4 µg/mL of PAP) were determined by comparing mean peak area of DIC/IS or PAP/IS samples to that of unextracted DIC or PAP standards in mobile phase at equivalent DIC or PAP level spiked with IS and expressed in percentage, respectively.

Stability of samples

Short-term stability of plasma samples was examined by supplementing blank plasma with appropriate amounts of working solutions of DIC and PAP to obtain 0.05, 0.25, 1.25, 5.0 µg/mL of DIC and 0.05, 0.2, 2.0, 6.0 µg/mL of PAP. Each sample was analyzed at room temperature for 48 h (every 4 h), including their residence time in an autosampler.

Table 2. Intra- and inter-day validation of the method (precision and accuracy) (n = 5).

Intra-day				Inter-day		
Added concentration (µg/mL)	Found concentration (µg/mL)	Precision (% RSD)	Accuracy (relative error, %)	Found concentration (µg/mL)	Precision (% RSD)	Accuracy (relative error, %)
DIC						
0.05	0.0477	7.6	4.6	0.0464	5.9	7.2
0.25	0.2395	3.3	4.2	0.2368	4.0	5.3
1.25	1.1588	4.4	7.3	1.1738	2.1	6.1
5	4.6752	4.5	6.5	4.8122	1.7	3.8
PAP						
0.05	0.0474	8.7	5.2	0.0482	7.6	3.6
0.2	0.1954	3.4	2.3	0.1952	4.5	2.4
2	1.8463	2.3	7.7	1.8823	5.2	5.9
6	5.4871	7.0	8.5	5.6401	4.8	6.0

The *long-term freezer stability* of DIC and PAP in rabbit plasma was assessed by analysis after 30 days of storage at -20°C. The concentration of the stability of the samples (0.05, 0.25, 1.25, 5.0 μg/mL for DIC and 0.05, 0.2, 2.0, 6.0 μg/mL for PAP) was compared to the mean of back-calculated values for the standards at the appropriate concentration from the first day of the long term stability testing.

The long-term freeze-thaw stability of the active substances at concentrations 0.05, 0.25, 1.25, 5.0 μg/mL for DIC and 0.05, 0.2, 2.0, 6.0 μg/mL for PAP in plasma was analyzed in triplicate after 1, 2 and 30 days of storage at -20°C.

Pharmacokinetic study

Animals

The experiments were carried out on randomly selected male Berg rabbits weighing 2.5-3.0 kg, purchased from the licensed breeder (Lisowski, Parczew, Poland). The animals were kept in cages (one per cage) on a 12-hour day/night cycle with free access to food and water. Each experimental group consisted of 9-12 animals. The experimental protocol was approved by the Local Ethics Committee at the Medical University of Lublin (license number 36/2007). All procedures were performed in compliance with the requirements of European convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS123, 1991).

Administration and sampling

Before the experiment, the rabbits were fasted for the period of 12 h but water was available.

On the first day of the test, the rabbits were divided into four groups and each group consisted of three animals. DIC and PAP used for intravenous administration were separately dissolved in sterile apyrogenic saline. The first group of animals was given a single intravenous injection of sterile apyrogenic physiological saline (as negative control), the second group was administered DIC at a dose of 12.5 mg/kg body weight (b.w.), the third group was injected with PAP at a dose of 5 mg/kg b.w. and the fourth group with DIC (12.5 mg/kg b.w.) and PAP (5 mg/kg b.w.). Intravenous administration was performed in the marginal ear vein of the rabbits at a volume of 0.5 mL/kg b.w. with sterile disposable syringes and needles.

The next day, other rabbits were divided into three groups and each group consisted of three animals. The first group was orally administered a tablet with DIC (12.5 mg/kg b.w.), the second group was given a tablet with PAP (5 mg/kg b.w.) and the third group a tablet composed of DIC (12.5 mg/kg b.w.) and PAP (5 mg/kg b.w.). The tablets were administered *via* an orogastric tube with 100 mL of water. Blood samples were withdrawn from the ear vein at different time intervals: 0, 0.5, 1, 2, 3, and 4 h. These intervals were chosen because the biological half-time of DIC and PAP is 1-2 h (3). In humans, dogs and pigs the terminal elimination half-lives ($t_{1/2â}$) of diclofenac have been reported to range from 1.1 to 2.4 h (9, 41, 42). The blood was collected into heparinized tubes. Plasma was separated and stored at -20°C till the analysis was carried out. All of the plasma samples were analyzed within a week after the separation.

After a lag period of 10 days, the animals were crossed over and the experiments were repeated. Finally, DIC and PAP were administered intravenously and orally in single and composed preparations six times (n = 6). The experiment was carried out at room temperature. All preparations were administered in the morning.

Calculations and statistics

The non-compartmental pharmacokinetic analysis of DIC and PAP concentrations in plasma *versus* time data were calculated using WinNonlin 1.1 software. The plasma concentration – time profiles following a single *i.v.* or *p.o.* doses were not adequately fitted with a compartmental model.

Statistical analysis was carried out using SAS 9.1.3 (SAS Institute, Cary, NC, USA). The data obtained were subjected to statistical analysis using one-way ANOVA and Student's *t*-test and a *p* value of < 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

HPLC method development

Different mobile phases were used to determine either DIC or PAP in biological fluids by HPLC (23-28, 34-36). There is no mobile phase for simultaneous determination of DIC and PAP in biological matrices. In our previous study (40) on simultaneous determination of DIC and PAP in composed tablets, the HPLC method with a mobile phase (methanol-water, 60 : 40, v/v) was developed. Bearing those studies in mind, we tried to achieve a proportion of solvents with an optimized separation of the active substances. It was not easy because the solubility of DIC and PAP is different (3). Various proportions of methanol-water solvents in mobile phase for determination of DIC in human plasma by HPLC method can be found in the literature (30, 43). In our study, methanol-water (55 : 45, v/v) was selected as the mobile phase for determination of

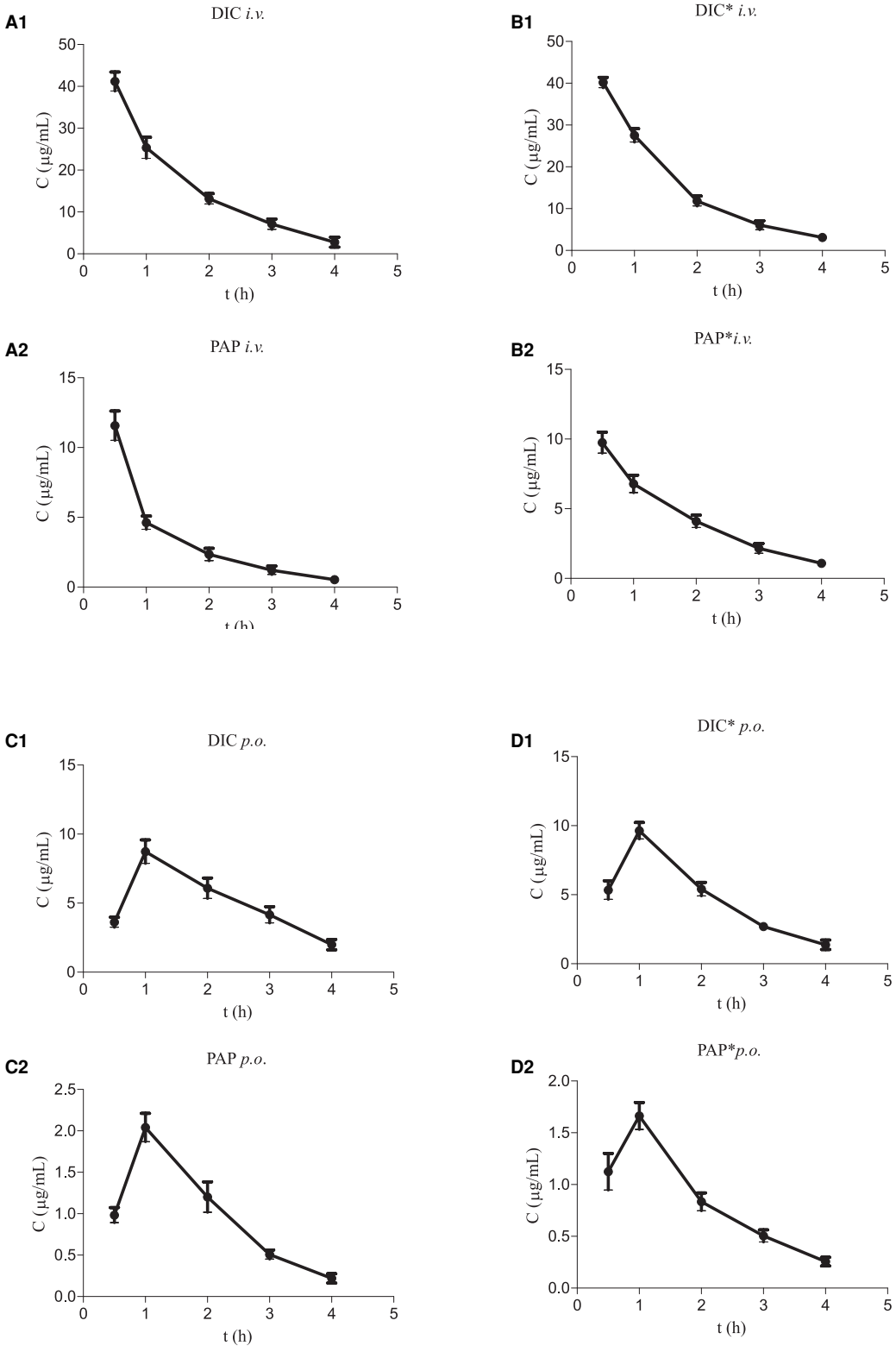

Figure 1. Representative chromatograms of: (A1) DIC (4 µg/mL), PAP (2 µg/mL) and phenacetin (IS) (6 µg/mL) standards at 280 nm and (A2) at 238 nm; (B1) blank rabbit plasma at 280 nm; (B2) blank rabbit plasma at 238 nm; (C1) rabbit plasma sample at 0.5 h after oral administration of composed tablet comprising DIC and PAP and spiked with IS at 280 nm and (C2) at 238 nm; (D1) DIC for LLOQ; (D2) PAP for LLOQ

DIC and PAP in rabbit plasma because of its very simple composition, and clear baseline separation of DIC, PAP and IS was obtained (Fig. 1).

A number of NSAIDs have been used as an internal standard for the analysis of DIC from the biological matrices (19, 21, 31). Several substances were tested as IS. There were very poor baseline separations of ibuprofen, naproxen, ketoprofen or indomethacin from DIC under the experimental conditions used in the present study. However, using phenacetin, clear line separation was achieved. Phenacetin seemed to be the most appropriate drug

Table 3A. Stability of DIC in rabbit plasma samples.

	Short-term stability (48 h) in an autosampler			
Concentration of DIC (μg/mL)	0.05	0.25	1.25	5.0
Mean	0.055	0.2402	1.37	5.31
SD	0.0013	0.0029	0.0351	0.0922
CV (%)	2.36	1.21	2.56	1.74
	Long-term freezer stability (30 days)			
Concentration of DIC (μg/mL)	0.05	0.25	1.25	5
Mean	0.048	0.2576	1.29	5.15
SD	0.0019	0.0057	0.0521	0.2138
CV (%)	3.96	2.21	4.04	4.15
	Long-term freeze-thaw stability after three cycles (-20°C, 30 days)			
Concentration of DIC (μg/mL)	0.05	0.25	1.25	5
% of the initial conc. of DIC after 30 days	97.43	98.32	97.12	98.89
SD	2.14	1.68	1.89	2.21
CV (%)	0.02	0.02	0.02	0.02

Table 3B. Stability of PAP in rabbit plasma samples.

	Short-term stability (48 h) in an autosampler			
Concentration of PAP (μg/mL)	0.05	0.2	2.0	6.0
Mean	0.053	0.2073	2.09	6.25
SD	0.001	0.0034	0.0321	0.0822
CV (%)	1.89	1.64	1.54	1.32
	Long-term freezer stability (30 days)			
Concentration of PAP (μg/mL)	0.05	0.2	2.0	6.0
Mean	0.0512	0.2155	2.11	6.17
SD	0.0023	0.0098	0.0821	0.2492
CV (%)	4.49	4.55	3.89	4.04
	Long-term freeze-thaw stability after three cycles (-20°C, 30 days)			
Concentration of PAP (μg/mL)	0.05	0.2	2.0	6.0
% of the initial conc. of PAP after 30 days	98.32	99.03	95.78	98.16
SD	2.5	1.98	2.08	2.41
CV (%)	0.03	0.02	0.02	0.02

Table 4. Pharmacokinetic parameters of DIC and PAP in single or composed formulations administered intravenously or orally.

Parameters	Intravenous				Oral			
	Single		Composed		Single		Composed	
	DIC	PAP	DIC	PAP	DIC	PAP	DIC	PAP
$AUC_{0\rightarrow\infty}$ (h·mg/L) (mean n = 6, ± SD)	83.88 ± 18.78	21.46 ± 5.71	80.34 ± 12.54	22.24 ± 4.65	24.32 ± 5.57	4.12 ± 1.02	21.52 ± 4.36	3.77 ± 0.66
MRT (h) (mean n = 6, ± SD)	1.30 ± 0.45	0.96 ± 0.33	1.32 ± 0.30	1.56 ± 0.38	2.68 ± 0.66	1.82 ± 0.19	2.16 ± 0.71	2.14 ± 0.30
CL (L/h) (mean n = 6, ± SD)	0.49 ± 0.13	0.78 ± 0.23	0.49 ± 0.07	0.74 ± 0.21	0.51 ± 0.09	0.79 ± 0.18	0.49 ± 0.11	0.72 ± 0.20
MAT (h) (mean n = 6, ± SD)					0.72 ± 0.20	0.86 ± 0.43	1.03 ± 0.83	0.76 ± 0.27
F (mean n = 6, ± SD)					0.30 ± 0.07	0.21 ± 0.11	0.28 ± 0.08	0.18 ± 0.07

Pharmacokinetic parameters of DIC and PAP, administered intravenously and orally showed no statistically significant difference between single or composed formulations ($p > 0.05$).

in the present assay and was chosen because it is stable and does not endogenously exist in plasma. Moreover, it did not interfere with the rabbit plasma sample and it was well separated from DIC and PAP.

Liquid-liquid extraction method was used for sample preparation. When it comes to extracting DIC from plasma after the acidification of the sample, hexane-isopropanol at different ratios (44, 45), acetonitrile (19, 46), and dichloromethane (30) were described in the literature. In our study, chloroform was used as solvent for extraction after acidification of plasma sample, because DIC and PAP are soluble in acidic medium and IS in chloroform. This solvent was adopted due to its high extraction efficiency and little interference.

HPLC method validation

Selectivity

No interference of endogenous and extraneous peaks with DIC, PAP and IS at their respective retention times at two wavelengths (RT_{DIC} = 2.87 min, RT_{IS} = 4.77 min and RT_{PAP} = 13.15 min at 280 nm and RT_{PAP} = 13.14 min, RT_{IS} = 4.76 min, RT_{DIC} = 2.85 min at 238 nm) in blank rabbit plasma was observed, as shown in Figure 1.

Sensitivity

The LLOQ of DIC and PAP in 1 mL rabbit plasma was observed to be 0.05 µg/mL.

The limit of detection (LOD) for DIC amounted to 0.015 µg/mL and for PAP to 0.02 µg/mL, based on a signal to noise ratio > 3.

Linearity of calibration curve

The method has a good linearity over the range of 0.05-5 µg/mL for DIC and 0.05-6 µg/mL for PAP. The mean regression equation from five replicate calibration curves was y = 0.1927 (± 0.0415) x + 0.0011 (± 0.00062) for DIC and y = 0.1449 (± 0.0267) x + 0.0033 (± 0.0017) for PAP. The square root of mean correlation coefficient (r^2) was 0.9974 for DIC and 0.9986 for PAP.

Precision and accuracy

The intra- and inter-day precision and accuracy validation with samples of DIC and PAP are presented in Table 2. In the range of 0.05-5 µg/mL DIC, the intra- and inter-day assay precision (RSD) varied from 3.3 % to 7.6% and from 1.7% to 5.9%, respectively. The intra- and inter-day accuracy (relative error) ranged from 4.2% to 6.5% and from 3.8% to 7.2%, respectively.

In the range of 0.05-6 µg/mL PAP, the intra- and inter-day assay precision (RSD) varied from 2.3% to 8.7% and from 4.5% to 7.6%, respectively. The intra- and inter-day accuracy (relative error) ranged from 2.3% to 8.5% and from 2.4% to 6.0%, respectively. The RSD of intra- and inter-day variability was less than 10%, which is within the limit

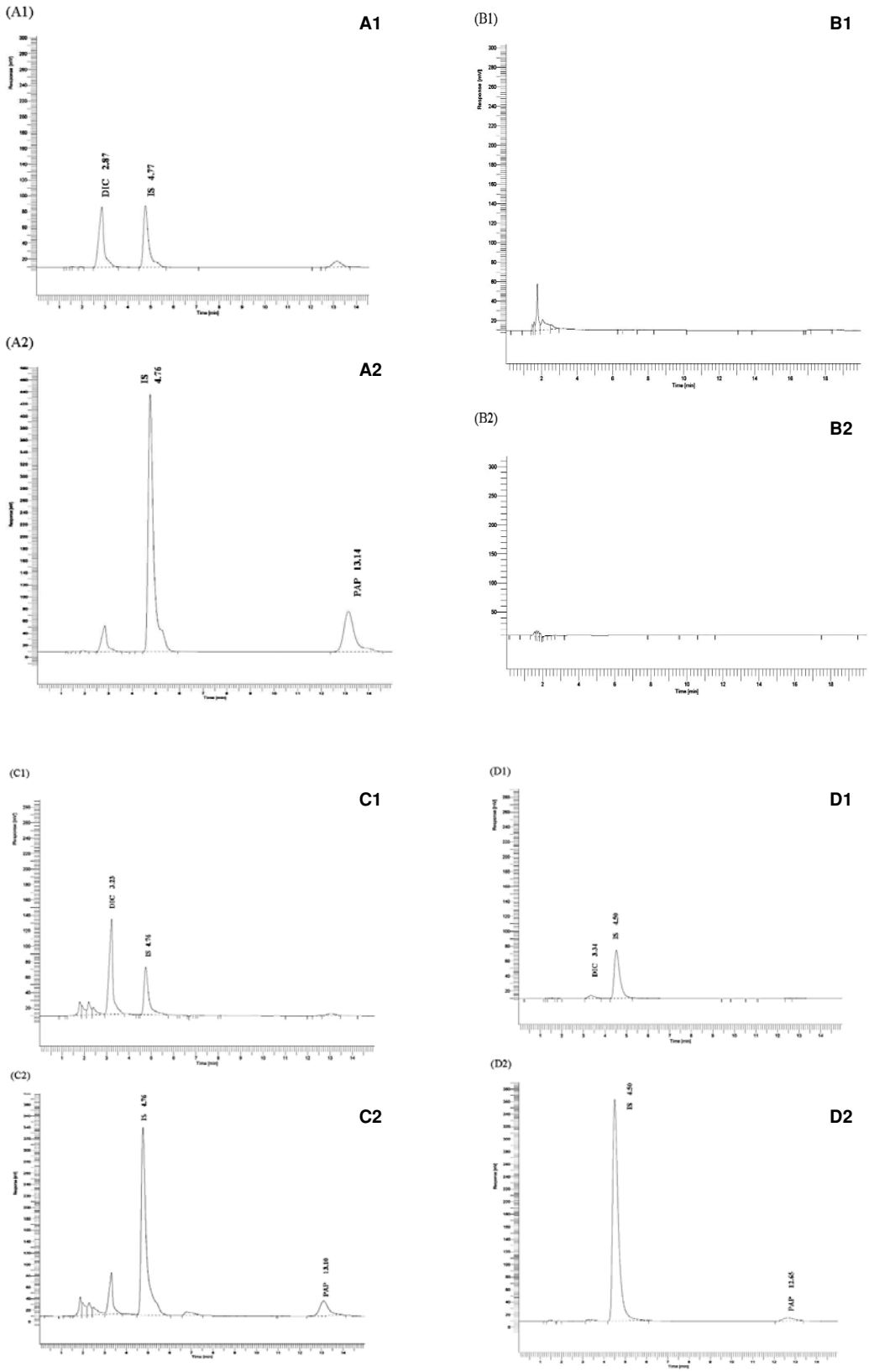

Figure 2. Mean plasma concentration-time profile (n = 6) for DIC and PAP in rabbit plasma after an intravenous (*i.v.*) or oral (*p.o.*) administration of single (DIC, PAP) or composed (DIC*, PAP*) formulations

of acceptability (± 15%) (24). These results indicate that this method had good precision and accuracy.

Recovery

The mean extraction recoveries of DIC were found to be 95.78% ± 2.98 of 0.05-5 µg/mL, PAP 93.09% ± 3.29 of 0.05-6 µg/mL and IS 95.93% ± 1.38 of 5-20 µg/mL, respectively. The recoveries were not dependent on the concentration and consequently resulted in good linearity of the calibration curves.

Evaluation of stability of samples

Stock solutions of DIC (250 µg/mL), PAP (100 µg/mL) and IS (100 µg/mL), and working solutions of DIC, PAP and IS were observed to be over 99% of the nominal concentrations after 5 days of storage at 4°C, compared with freshly prepared solutions. Similarly, working solutions of DIC, PAP and IS were over 99 % of the nominal concentrations after 20 days of storage at -20°C.

Short-term stability of active substances in plasma were investigated at room temperature for 48 h. After the storage, the coefficients of variation (CV, %) values varied from 1.21 to 2.56 for DIC solutions and from 1.32 to 1.89 for PAP concentrations.

Long-term freezer stability of plasma samples (after 30 days of storage at -20°C) CV values for analyzed DIC samples: 0.05, 0.25, 1.25, 5.0 µg/mL amounted to 3.96, 2.21, 4.04 and 4.15%, respectively. PAP (CV) values amounted to 4.49, 4.55, 3.89 and 4.04% for concentrations: 0.05, 0.2, 2.0, 6.0 µg/mL, respectively.

After storage in *long-term freeze-thaw stability* for three cycles (-20°C, 30 days) the drugs were regarded as stable if more than 90% was intact at the end of the study period. The amount of the initial concentration of DIC and PAP remaining after this time was: 97.43 ± 2.14%, 98.32 ± 1.68%, 97.12 ± 1.89%, 98.89 ± 2.21% for DIC concentrations of 0.05, 0.25, 1.25 and 5.0 µg/mL, respectively, and 98.32 ± 2.5%, 99.03 ± 1.98%, 95.78 ± 2.08% and 98.16 ± 2.41% for PAP concentrations of 0.05, 0.2, 2.0 and 6.0 µg/mL respectively.

The results from the stability tests (Tables 3 A and B) indicated that DIC and PAP were stable under the conditions studied.

Pharmacokinetic study

The mean plasma concentration-time profiles of DIC and PAP are shown in Figure 2. The pharmacokinetic parameters of DIC and PAP were calculated by non-compartmental analysis and are presented in Table 4. More than 99% of diclofenac is bound to plasma proteins (3, 9) and papaverine is about 90% (3), therefore, we expected changes in the pharmacokinetic parameters of substances after their co-administration. As compared with the pharmacokinetic parameters, such as $AUC_{0\to\infty}$, MRT and CL of DIC or PAP administered intravenously and orally, after single dose there was no statistically significant difference between single or compound formulations ($p > 0.05$). The MAT of DIC and PAP administered as single or compound formulations were calculated to be over 1 h and these MAT values presented no statistically significant difference between the formulations ($p > 0.05$). Similarly, after the oral administration of DIC to the rabbits, the mean MAT was equal to 1.24 h (47). Diclofenac penetrates to synovial fluid where concentrations may persist even when in plasma concentrations fall. Diclofenac is metabolized by cytochrome P450 by phase I hydroxylation and by phase II conjugation with glucuronic acid and the amino acid taurine. Diclofenac is extensively metabolized in camels as well as in humans, goats and cattle, and this appears to be mediated by cytochrome P450 2C subfamily (48, 49).

Drug-protein binding has an influence on the distribution equilibrium of drugs. Only the free, non-protein bound fraction of drug can leave the circulatory system and diffuse into tissue. The equilibrium between free drug can be maintained over a relatively long period of time because of the dissociation of the drug-protein complex. The transport function of plasma proteins is of importance for drugs of low solubility in water. Plasma protein binding is of significant influence on the distribution equilibrium if the drug is polar and therefore diffuses slowly into tissue. If, in addition, such a substance has a high affinity for plasma proteins, displacement from its protein binding sites may result in a change of distribution equilibrium and an altered pharmacologic response (50).

The $AUC_{0\to\infty}$ values of DIC or PAP after intravenous injection were higher than after oral administration. It was shown that bioavailability of (F) DIC and PAP after oral administration was decreased to over 30% for DIC and 20% for PAP, but for some rabbits bioavailability amounted to over 43% for both DIC and PAP. When DIC is orally administered, it is almost completely absorbed due to the fact that it is subject to first-pass metabolism. In this way about 50% of the drug reaches the systemic circulation in the unchanged form (3).

The metabolic tracts of both of the substances showed that it is possible that a simultaneous admin-

istration of DIC and PAP has no influence on their pharmacokinetics.

In our study, oral administration of a tablet comprising DIC and PAP did not change the main pharmacokinetic parameters of the active substances after single dose. Similarly, administration of diclofenac and misoprostol in one composed tablet did not change the pharmacokinetics of these two drugs (4).

CONCLUSION

Tablets comprising DIC and PAP were prepared and all of tablets fulfilled pharmacopoeal requirements such as average weight, hardness and assay of drug content. A HPLC-UV method with LLE extraction was developed and validated for the quantitative determination of DIC and PAP in rabbit plasma. The LLOQ of the method was 0.05 μg/mL and the sensitivity was compared with previously reported HPLC-UV methods using LLE (21) and protein precipitation (19). The proposed method was successfully applied to determination of DIC and PAP, after injection and oral administration to rabbits, for use in pharmacokinetic study. A non-compartmental pharmacokinetic analysis showed that oral administration of composed tablet comprising DIC and PAP did not change the main pharmacokinetic parameters such as MRT, MAT, CL and bioavailability of active substances compared with a single administration of DIC and PAP after single dose.

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